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Diacetylmorphine degradation to 6-monoacetylmorphine and morphine in cell culture: implications for in vitro studies

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Abstract

Diacetylmorphine deacetylates rapidly to 6-monoacetylmorphine and then to morphine. The immunomodulatory effects of diacetylmorphine are under investigation by several groups utilising various methods including in vitro cell culture; however, diacetylmorphine stability under these conditions is unknown. The aim of this study was to quantify diacetylmorphine degradation under cell culture conditions and to determine the mechanism by which this occurs. Diacetylmorphine degradation in a mouse splenocyte mitogenesis assay was investigated. Morphine and 6-monoacetylmorphine were quantified using HPLC with UV detection. After 6 h, approximately 73% of diacetylmorphine had been hydrolysed in the presence of cells. The half-life of diacetylmorphine was 1.4 h in cell media alone and 1.2–2.2 h in incubations containing cells, while the half-life of 6-monoacetylmorphine was 3.1 h in cell media alone and 0.99–1.2 h in incubations containing cells. 6-Monoacetylmorphine and morphine formation were found to be dependent on incubation time and diacetylmorphine concentration, and were not dependent on esterase activity, mitogen concentration, presence of erythrocytes and cell media evaporation. Only morphine formation was dependent on lymphocyte concentration. 6-Monoacetylmorphine formation was independent of cells and appeared to be due to the conditions of the cell culture (pH and temperature), while morphine formation was dependent to a greater extent on cells, but independent of esterase activity. The study highlights the limitations of conclusions made in previous studies which have not recognised diacetylmorphine instability.

Keywords: Diacetylmorphine degradation; Heroin; Morphine; 6-Monoacetylmorphine; Tissue culture

1. Introduction

Diacetylmorphine (heroin or 3,6-diacetylmorphine) is a highly addictive opioid agonist whose use accounts for many drug-related illnesses, deaths and social problems (Ryan and White, 1996). Diacetylmorphine rapidly deacetylates (half-life of 3 min) in vivo to 6-monoacetylmorphine (Fig. 1), which is further rapidly deacetylated to morphine (Inturrisi et al., 1984; Rentsch et al., 2001). In vivo, these reactions are catalysed by serum butyrylcholinesterase (diacetylmorphine to 6-monoacetylmorphine) (Lockridge et al., 1980; Salmon et al., 1999) and erythrocyte acetylcholinesterase (diacetylmorphine to 6-monoacetylmorphine to morphine) (Salmon et al., 1999). In vitro, the stability of diacetylmorphine is significantly affected by pH, temperature and light (Barrett et al., 1992; Wijesekera et al., 1994). Small changes in these conditions (pH or temperature) can cause increased spontaneous diacetylmorphine hydrolysis to 6-monoacetylmorphine, and 6-monoacetylmorphine to morphine.

The pharmacological activity of diacetylmorphine has been attributed to its metabolites, 6-monoacetylmorphine and morphine, and morphine's metabolites all acting on opioid receptors (White and Irvine, 1999). Recently however, a specific diacetylmorphine receptor has been identified by which diacetylmorphine alone could produce analgesia (Brown et al., 1997; Rossi et al., 1997). In light of the instability of diacetylmorphine, it is difficult to isolate its effects in in vitro systems and subsequently attribute these to diacetylmorphine and not to its active metabolites. For example, a number of studies have investigated the immunomodulatory behaviour of diacetylmorphine in vitro (Perez-Castrillon et al., 1992; Thomas et al., 1995b). In the

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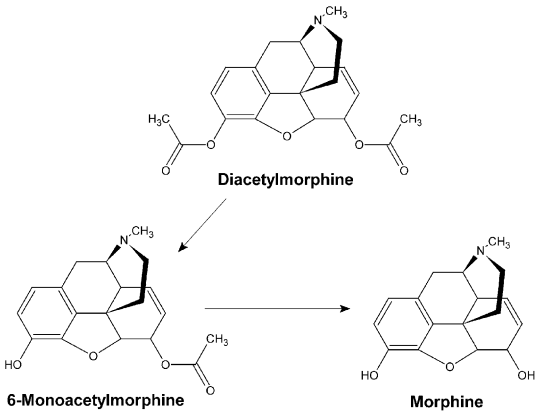

Fig. 1. Diacetylmorphine degradation pathway.

cell culture conditions employed by Thomas et al. (1995b), diacetylmorphine was incubated for more than 72 h at 37 °C in media which is subject to pH change and the possible exposure to various esterases, all of which are likely to cause increased diacetylmorphine degradation. 6-Monoacetylmorphine, morphine and morphine's metabolites (morphine-3-glucuronide and morphine-6-glucuronide) have significant immunomodulatory effects (House et al., 1997; Pacifici et al., 2000; Thomas et al., 1995a). Hence, the effects of diacetylmorphine itself cannot be determined unless the extent of degradation and the primary catalyst(s) of the degradation processes are known and taken into consideration. There are no data on the degradation of diacetylmorphine in tissue culture. The aim of this study was to quantify the extent of diacetylmorphine degradation to 6-monoacetylmorphine and subsequently to morphine in cell culture (splenocyte mitogenesis assay), and to determine the mechanism by which diacetylmorphine hydrolyses under these conditions.

2. Materials and methods

2.1. Chemicals

RPMI 1640 with HEPES modification, L-glutamine, penicillin–streptomycin solution (10,000 units penicillin and 10 mg streptomycin per ml), lipopolysaccharide from *Salmonella typhimurium*, phytohemagglutinin, concanavalin A, sodium dodecyl sulphate phenylmethylsulfonyl fluoride and 1,5-*bis*-4-allydimethylammoniumphenyl pentane-3-one dibromide (BW284c51) were purchased from Sigma (St. Louis, MO, USA). Foetal calf serum was obtained from Trace Scientific (Melbourne, Vic., Australia). Sterile tissue culture 96-well plates were obtained from Sarstedt (Newton, NC, USA). Eserine was obtained from BDH Australia (Smithfield, N.S.W., Australia) and diacetylmorphine base from the National Analytical Reference Laboratory of the Australian Government Analytical Laboratories (Pymble, N.S.W., Australia). 6-Monoacetylmorphine was a gift from Noel Sims (Forensic Science Centre, Adelaide, Australia). Codeine phosphate was obtained from F.H. Faulding (Adelaide, S. A., Australia) and morphine hydrochloride from McFarlane Smith (Edinburgh, UK). All other reagents and chemicals were obtained from commercial sources and were of analytical-grade quality.

2.2. Preparation of solutions

Dissolving diacetylmorphine base directly into Milli Q water or an equimolar solution of hydrochloric acid caused spontaneous diacetylmorphine degradation of 10–15%. To prevent this degradation, diacetylmorphine base was dissolved in pH 5.0 phosphate buffer (monopotassium phosphate, 65.9 mM and disodium phosphate, 0.8 mM). Once in solution, diacetylmorphine was stored at −80 °C until required. Minimal diacetylmorphine degradation during storage was observed (<1% in over 3 months). 6-Monoacetylmorphine and morphine solutions in Milli Q water were stored at −80 °C until required with minimal degradation during storage (<1% in over 3 months).

2.3. Animals

Ethics approval to conduct the studies was obtained from the Adelaide University Animal Ethics Committee. Male balb/c mice, 6–8 weeks old, were purchased from Central Animal Supplies (Waite campus, Adelaide University, Adelaide, Australia) and used as donors of splenocytes for use in cell culture. Animals were provided with standard rodent feed and water ad libitum. Animals were housed in a standard 12 h light–dark cycle (starting at 7 a.m.) under constant room temperature of 22 ± 2 °C.

2.4. Lymphocyte and multiwell plate preparation

Aseptic techniques were used during the preparation of the lymphocytes. Mice were sacrificed by carbon dioxide asphyxiation followed by prompt removal of the spleen. The spleen was prepared as a single cell suspension by massaging and washing through a nylon mesh into a 10 ml tube with 10 ml of RPMI 1640 (HEPES modification, 0.3 mg ml^{-1} L-glutamine and 5 ml penicillin–streptomycin solution per l). The cells were centrifuged at 4 °C for 5 min at 1000 rpm; the supernatant was discarded and the cells resuspended in 5 ml of enriched RPMI 1640 (RPMI 1640 enriched with 20% foetal calf serum). The number of viable lymphocytes in the suspension was counted using Trypan blue and a haemocytometer.

2.4.1. Incubation time-dependency study

Each well of the multiwell plates contained 100 μl of the cell suspension (5×10^6 cell ml^{-1} diluted in enriched media), 50 μl of diacetylmorphine (final concentration of

50 μM) and 50 μl of mitogen (final concentrations: concanavalin A, 2.5 μg ml^{-1}; phytohemagglutinin, 15 μg ml^{-1} and lipopolysaccharide, 25 μg ml^{-1}) or cell media. Wells containing no cells were also prepared by adding 100 μl enriched media. Plates were incubated at 37 °C, 5% CO_2 in a humidified incubator (Thermoline, N.S.W., Australia) for 0, 1, 4, 6, 24, 30 and 53.5 h, at which time, samples were taken for analysis.

2.4.2. Esterase inhibitor study

Each well of the multiwell plates contained 100 μl of the cell suspension (5×10^6 cell ml^{-1} diluted in enriched media), 50 μl of diacetylmorphine (final concentration of 50 μM), 25 μl of mitogen (final concentrations: concanavalin A, 2.5 μg ml^{-1}; phytohemagglutinin, 15 μg ml^{-1} and lipopolysaccharide, 25 μg ml^{-1}) and 25 μl of the esterase inhibitors: eserine (physostigmine, 200 μM), phenylmethylsulfonyl fluoride (500 μM) or BW284c51 (10 μM). Plates were incubated for 5 h as previously described, following which samples were taken for analysis.

2.4.3. Diacetylmorphine concentration-dependency study

Each well of the multiwell plates contained 100 μl of the cell suspension (5×10^6 cell ml^{-1} diluted in enriched media), 50 μl of diacetylmorphine (final concentrations of 5, 10, 20, 40, 80 and 160 μM) and 50 μl of mitogen (final concentrations: concanavalin A, 2.5 μg ml^{-1}; phytohemagglutinin, 15 μg ml^{-1} and lipopolysaccharide, 25 μg ml^{-1}) or cell media. Plates were incubated for 5 h as previously described, following which samples were taken for analysis.

2.4.4. Mitogen concentration-dependency study

Each well of the multiwell plates contained 100 μl of the cell suspension (5×10^6 cell ml^{-1} diluted in enriched media), 50 μl of diacetylmorphine (final concentration of 50 μM) and 50 μl of mitogen (final concentrations—concanavalin A: 1, 2.5 and 10 μg ml^{-1}; phytohemagglutinin: 5, 15 and 50 μg ml^{-1} and lipopolysaccharide: 5, 25 and 50 μg ml^{-1}) or cell media. Plates were incubated for 5 h as previously described, following which samples were taken for analysis.

2.4.5. Erythrocyte lysis study

Cells were prepared as before except that preferential lysis of the erythrocytes was carried out after the first wash step. Cells were resuspended in 1 ml of media followed by the addition of 10 ml of lysis buffer (ice cold 1 ml of 20.56 g l^{-1} Tris base (pH 7.65) and 9 ml of 0.83% NH_4Cl in H_2O, mixed just prior to addition to cells). The suspension was placed on ice for 4 min, centrifuged, followed by a wash and cell count as before. Each well of the multiwell plates contained 100 μl of the cell suspension (5×10^6 cell ml^{-1} diluted in enriched media), 50 μl of diacetylmorphine (final concentration of 50 μM) and 50 μl of mitogen (final concentrations: concanavalin A, 2.5 μg ml^{-1}; phytohemagglutinin, 15 μg ml^{-1} and lipopolysaccharide, 25 μg ml^{-1}) or cell media. Plates were incubated for 5 h as previously mentioned, following which samples were taken for analysis.

2.4.6. Cell number dependency study

Each well of the multiwell plates contained 100 μl of the cell suspension (5×10^4, 5×10^6 and 5×10^8 cells ml^{-1} cell suspensions diluted in enriched media), 50 μl of diacetylmorphine (final concentration of 50 μM) and 50 μl of cell media. Plates were incubated for 5 h as previously described, following which samples were taken for analysis.

2.4.7. Splenocyte suspension lysis study

To investigate the effect cellular constituents have on the rate of diacetylmorphine degradation, the whole splenocyte suspension was lysed with 1 mM HCl. The lysis of splenocytes was performed after the cell count by adding 1 ml of 1 mM HCl (final volume of 6 ml). The suspension was then centrifuged as before and diluted to a theoretical cell concentration of 5×10^6 cells ml^{-1} using the previous cell count. One hundred microlitres of the suspension was added to the multiwell plates as stated previously, along with 50 μl of diacetylmorphine (final concentration of 50 μM) and 50 μl of cell media. Plates were incubated for 5 h as previously described, following which samples were taken for analysis.

2.4.8. Cell media evaporation study

The extent of evaporation during the period of incubation was assessed by incubating a plate for 53.5 h, each well of which contained 50 μl of internal standard (codeine, 100 μg ml^{-1}) and 150 μl media. Following the incubation, 100 μl was taken from 12 wells, without the addition of internal standard, assayed and compared with a freshly prepared control.

2.5. Sample preparation for HPLC quantification

Samples were collected by adding 50 μl of internal standard (codeine, 100 μg ml^{-1}) to the wells, followed by removal of a 175 μl aliquot of the solution, which was then transferred to 10 ml polypropylene tubes containing 300 μl of 20% Na_2CO_3. Dichloromethane (4 ml) was added to the tubes followed by 10 min of rotary mixing and centrifugation at $1700 \times g$ for 10 min. The aqueous layer was aspirated and the remaining organic phase was transferred to a new 10 ml tube containing 200 μl of 0.1 M HCl. The tubes were rotary mixed for 10 min followed by centrifugation at $1700 \times g$ for 10 min. Approximately 150 μl of the acid bubble was removed and prepared for auto injection onto the HPLC system.

2.6. Quantification of morphine and 6-monoacetylmorphine

The HPLC system comprised a SIL-9A autosampler (Shimadzu, Kyoto, Japan), LC-6A pump (Shimadzu), Jasco UVIDEC-100-V UV spectrophotometer (Japan

Spectroscopic, Tokyo, Japan) and a C-R6A integrator (Shimadzu). The stationary phase consisted of a spherisorb 5μ C18 (15 cm × 4 mm) column, and an in-line pre-column cartridge (10 mm Alltima C18 5U, Alltech, IL, USA) was positioned ahead of the column. The mobile phase contained 30% acetonitrile, 0.01 M KH_2PO_4 and 230 mg l^{-1} sodium dodecyl sulphate adjusted to pH 2.3 with orthophosphoric acid. The mobile phase was filtered and subsequently sonicated for a minimum of 15 min. The flow rate through the column was 1.5 ml min^{-1} with absorbance measured at 210 nm (detector range 0.01 ABU). No interfering peaks were observed in the chromatography. A calibration curve of 10 standards was prepared consisting of morphine (1.4, 2.6, 5.3, 7.0, 8.8, 10.5, 17.5, 35.0, 52.6 and 70.1 μM) and 6-monoacetylmorphine (7.5, 10, 15, 20, 30, 40, 50, 60, 80 and 100 μM). The actual concentrations of the standards were four times greater, as they underwent a one in four dilution in the incubation (50 μl standard, 150 μl RPMI 1640). The samples were stored at −80 °C when not in use. The calibration curve samples were extracted at time zero with the time zero diacetylmorphine samples.

2.7. Data analysis

During the extraction process, diacetylmorphine spontaneously degraded due to the alkaline conditions of the extraction buffer. A time zero extraction sample was prepared to determine the degradation due to this extraction procedure alone. This degree of degradation was then subtracted from all subsequent data points resulting in the amount of morphine or 6-monoacetylmorphine formed due to the cell culture conditions alone. The concentration of diacetylmorphine was calculated by summing the molar concentrations of morphine and 6-monoacetylmorphine and subtracting this value from the initial concentration of diacetylmorphine (50 μM).

The rates of diacetylmorphine degradation to 6-monoacetylmorphine (k_1) and subsequently to morphine (k_2) were determined using the following equations:

$$\frac{dA}{dt} = -k_1 A + k_1 x$$

$$\frac{dB}{dt} = k_1 A - k_1 x - k_2 B + k_2 y$$

$$\frac{dC}{dt} = k_2 B - k_2 y$$

where A, B and C are diacetylmorphine, 6-monoacetylmorphine and morphine concentrations, respectively, and x and y are constants which allow optimal modelling since the degradations of diacetylmorphine and 6-monoacetylmorphine were not complete. The model was fitted to the data calculated for k_1 and k_2 using MicroMath Scientist for Windows version 2.0 (MicroMath, MI, USA).

Half-life was calculated by dividing ln(2) with the respective rate constants. Area under the curve and statistical calculations were performed using Prism 3.02 (GraphPad, San Diego, CA, USA). Statistical significance was assessed using a two-way analysis of variance with Bonferroni post hoc test. Significance was set at $P < 0.05$.

3. Results

Morphine and 6-monoacetylmorphine formation from diacetylmorphine increased significantly ($P < 0.0001$) in the presence of cells (mitogen-treated or untreated) from 4 h compared to RPMI 1640 alone (Fig. 2). Morphine and 6-monoacetylmorphine formation peaked at 6 h; thereafter, minimal increase in degradation occurred (Fig. 2). Phytohemagglutinin was the only mitogen which caused a significant ($P = 0.036$) increase in morphine formation compared to cells alone (Fig. 2), while there was no significant difference ($P > 0.36$) between cell treatments for 6-monoacetylmorphine. These differences were also observed in the area under the curve data for morphine concentrations (no cells, 488 μM h^{-1}; cells, 924 μM h^{-1}; lipopolysaccharide, 942 μM h^{-1}; concanavalin A, 966 μM h^{-1} and phytohemagglutinin, 1049 μM h^{-1}) and 6-monoacetylmorphine (no cells, 327 μM h^{-1}; cells, 494 μM h^{-1}; lipopolysaccharide, 676 μM h^{-1}; concanavalin A, 716 μM h^{-1} and phytohemagglutinin, 727 μM h^{-1}).

Total diacetylmorphine degradation, calculated from morphine and 6-monoacetylmorphine concentrations, peaked at 6 h with 73% of the initial diacetylmorphine

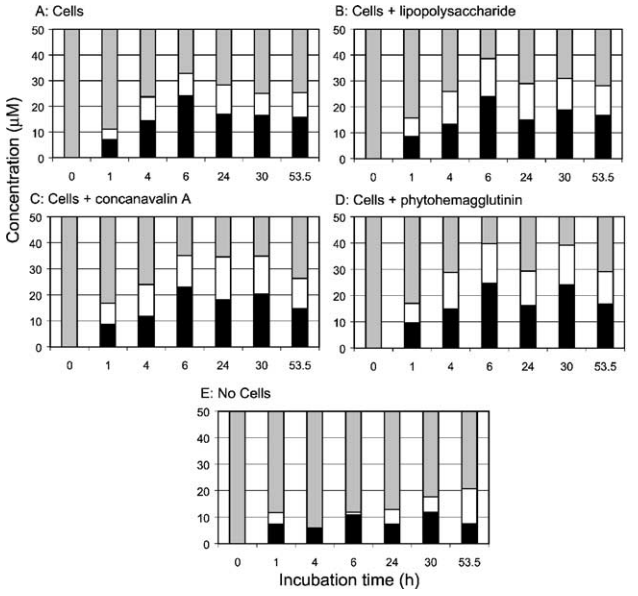

Fig. 2. Time-dependent degradation of diacetylmorphine (▩) to morphine (■) and 6-monoacetylmorphine (□) in cell culture under different conditions. (A) Cells; (B) cells + lipopolysaccharide (25 μg ml^{-1}); (C) cells + concanavalin A (2.5 μg ml^{-1}); (D) cells + phytohemagglutinin (15 μg ml^{-1}); (E) no cells. Initial diacetylmorphine concentration is 50 μM.

(50 μM) converted to morphine (~ 65%) and 6-monoacetylmorphine (~ 35%) (Fig. 2). The half-lives of diacetylmorphine and 6-monoacetylmorphine degradations were 1.4 and 3.1 h, respectively, in cell media alone. Coefficient of determination (r^2) values ranged from 0.97 for phytohemagglutinin to 0.99 for cells. The half-lives of diacetylmorphine and 6-monoacetylmorphine degradation were similar in the incubations containing cells, ranging from 1.2 (cells alone) to 2.2 h (concanavalin A) for diacetylmorphine and 0.99 (lipopolysaccharide) to 1.2 h (cells alone) for 6-monoacetylmorphine.

There was no significant evaporation ($P>0.74$) in the cell culture media during the incubation (data not shown). Mitogens alone (no cells) or the presence of splenocyte lysate did not increase the rate of diacetylmorphine degradation in RPMI 1640 (data not shown).

As the original diacetylmorphine concentration increased in the cell culture media, formation of morphine and 6-monoacetylmorphine increased linearly ($r^2>0.94$) over a diacetylmorphine concentration range of 5–160 μM after 5 h incubations. Morphine formation was related to increasing cell number after a 5 h incubation, with 5×10^7 cells causing the greatest degradation, while 6-monoacetylmorphine formation was not related to cell number. The use of esterase inhibitors or the removal of erythrocytes had no significant effect ($P>0.18$ and $P>0.22$, respectively) on diacetylmorphine degradation.

4. Discussion

This study was undertaken to quantify diacetylmorphine degradation in cell culture and to attempt to determine the mechanism by which this occurs, thus enabling future investigations of the specific effects of diacetylmorphine in vitro to be firmly established. Morphine and 6-monoacetylmorphine were detected in the cell culture media after just 1 h when incubated at 37 °C. Up to 80% of diacetylmorphine had been hydrolysed after 6 h in some of the conditions used in this study. The degradation of diacetylmorphine was significantly affected by the presence of cells as determined from the area under the curve data. Interestingly, the half-life of diacetylmorphine was similar in incubations with or without cells, while the half-life of 6-monoacetylmorphine was reduced by the presence of cells. Therefore, diacetylmorphine degradation is dependent on the conditions of the cell culture and not on the presence of cells. This is supported by the findings that esterase inhibitors, mitogen concentration, removal of erythrocytes and reduction of the lymphocyte concentration in the cell culture had no effect on 6-monoacetylmorphine formation.

Morphine formation is dependent to a greater extent on the presence of cells, since the half-life of 6-monoacetylmorphine without cells was greater than with cells, and morphine formation could be reduced with lower lymphocyte concentrations. However, this reaction does not appear to be mediated by esterases since the nonselective esterase inhibitors, eserine and phenylmethylsulfonyl fluoride (Zhang et al., 1999), and selective erythrocyte acetylcholinesterase inhibitor BW284c51 (Salmon et al., 1999) had no effect on morphine formation. Taken together, these results suggest that the major cause of 6-monoacetylmorphine degradation was nonenzymatic and possibly due to reactions or bi-products associated with lymphocyte activity.

Barrett et al. (1992) estimated the half-life of diacetylmorphine to be 32.9 h at 37 °C, pH 7.4 (phosphate buffer). The half-life of diacetylmorphine obtained in the current study in RPMI 1640 cell media (37 °C, assumed pH of 7.4) alone was 1.4 h, significantly shorter than that previously found for similar pH and temperatures (Barrett et al., 1992; Poochikian and Cradock, 1979). However, Barrett et al. (1992) found that very small increases in pH resulted in a significant decrease in the half-life of diacetylmorphine (pH 8.0, half-life of 8.3 h). A small increase in the pH of the cell culture media and the differences in the complexity of the media compared to the phosphate buffer may account for the difference found in diacetylmorphine's half-life.

The conclusions drawn from previous in vitro tissue culture studies investigating the effects of diacetylmorphine per se (Perez-Castrillon et al., 1992; Thomas et al., 1995b) have not taken into account the presence of morphine and 6-monoacetylmorphine in their experimental systems. Even though diacetylmorphine is rapidly metabolised to its active metabolites in vivo, the pharmacodynamic effects of diacetylmorphine alone must be elucidated in order to better understand the properties of this compound over those of morphine and 6-monoacetylmorphine. This study has highlighted the difficulty of using unstable compounds such as diacetylmorphine in cell culture. Unless precautions are taken to limit the degradation of diacetylmorphine, future studies which use diacetylmorphine in cell culture must acknowledge that significant quantities of morphine and 6-monoacetylmorphine are formed and must not assume that the responses observed are due entirely to diacetylmorphine alone. At present, there appears to be no straightforward method to circumvent diacetylmorphine degradation; therefore, shorter exposure times may be needed for use in combination with media replenishment to avoid accumulation of diacetylmorphine's active degradation products in the media.

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